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The goal of this research project is (a) designed to improve the quality of life by exploiting differences in the biochemical pharmacology of methotrexate (MTX) in MCF-7 breast cancer cells versus normal tissues and (b) provide one clear basis for intracellular rescue of only host cells from MTX toxicity when high dose MTX is used in combination with 5-fluorouracil (5-FU). The major findings are: (1) High dose MTX toxicity is reduced by a (priming - and non-toxic -)5-FU dose. 5-FU reverses high dose MTX acute toxicity on bone marrow. The erythroid cells appear to be more sensitive than the myeloid cells to the acute adverse effect of MTX. (2) A 50% reduction in the priming-dose of 5-FU was effective in providing marked protection against weight loss from an acute MTX dose 6 times the lethal dose. (3) Cytotoxicity to MCF-7 human breast cells may be the resultant of higher intracellular of methotrexate polyglutamate (MTX PGs) than MTX.

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Donnell Bowen 9/30/97

PI - Signature Date

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## Introduction

Background and Accomplishments of This Laboratory

Research activities in this laboratory have led to the development of a method whereby selectivity between cancer cells and normal cells (hematopoietic and gastrointestinal) may occur when methotrexate (MTX) is combined with the fluoropyrimidines, 5-fluorouracil (5-FU) and 5-fluorodeoxyuridine (5-FUdR). In vivo studies reported from this laboratory indicated that the administration of MTX after a *priming - and non-toxic dose of 5-FU enhanced the antitumor activity of MTX while decreasing its (MTX) toxicity (1,2). The in vivo studies have been extended to a Phase I study in which cancer patients received up to 1600 mg / $m^2$ of MTX without toxicity and / or lethality (3,4) (see patient data in appendix) -- 200 mg / $m^2$ of MTX is reported to be lethal (5). In vitro studies from this laboratory suggest that cells a) pretreated with 5-FU or 5-FUdR before MTX or b) MTX exposed simultaneously to either 5-FU or 5-FUdR conserved reduced folates and decreased MTX effects on DNA, RNA and protein synthesis (6,7). Further, these studies (in vitro and in vivo) suggest that reduced folates should be conserved in tumor and normal cells when fluoropyrimidines preceded MTX administration. However, the existence of MTX as a) MTX-polyglutamates (MTXPGs) and the monomer only in tumor cells and b) a monomer or monoglutamate (MTX) in normal cells. For example, MTX and MTXPGs have been found in tumor cells but only MTX or very little MTXPGs appeared in the bone marrow (8). By conserving cellular reduced-folates with fluoropyrimidines, sufficient levels of reduced-folates are present to protect normal cells against MTX, but insufficient levels to protect tumor cells against MTX and MTXPGs' depletion of reduced-folates.

Purpose and Scope of Subject Matter

The goal of this project is to use our previous experience to investigate research issues in protocol devlopment which impact upon the quality of life in breast cancer patients. There is a consensus that quality of life is amultidimensional construct, including at a minimum, disease and treatment related symptoms. Hence, this research project is (a) designed to improve the quality of life by exploiting differences in the biochemical pharmacology of MTX in MCF-7 and MDA-MB-436 breast cancer cells versus normal tissues and (b) provide one clear basis for intracellular rescue of only host cells from MTX toxicity when high dose MTX is used in combinatiion with 5-FU. Hence, an analysis of MTX and MTXPGs levels in MCF-7 breast cancer cells and subsequent effects on normal cells (bone marrow) were determined. Studies are in progress to define the optimal dose of MTX with respect to 5-FU by escalating the MTX dose. The parameters assessed are weight loss and survival.

**Body**

Experimental Methods, Assumptions, and Procedures

Animals

Female Balb/c mice weighing 18-22 g (approximate age 4-6 weeks) were obtained from Charles River Breeding Laboratories, Wilmington , MA. Upon arrival, mice were randomized and quarantined for at least one week. Animals were housed 5 per cage in a light (12 h/day), temperature (24°C), and humidity (60%) controlled room. Mice were given food and water ad libitum.

Toxicity Studies

MTX, at a dose level of 250 mg/kg, was given as a single i.p. injection either alone or various combinations of 25 mg/kg of 5-FU. 0.9% NaCl (sterile) was administered as a vehicle control. Animals surviving 3-14 days after a priming dose of 5-FU administered 2h prior to MTX, MTX given 2h prior to 5-FU, MTX, 5-FU, and control were anesthetized and blood collected by cardiac puncture in tubes containing EDTA for erythrocyte and leukocyte determination. After euthanasia, bone marrow was harvested by splitting the femur with a no. 11 scapel blade. Direct imprints of the exposed bone marrow cells were made on glass slides. Glass slides were the fixed in a methanolic solution, stained with an azo dye and followed by equilibration in an alkaline solution of the azo dye. The myeloid-erythroid ratio from bone marrow was determined by counting a minimum of 500 cells. In separate studies from above, MTX at a dose level of 1250 mg/kg (6 times the lethal dose) and 5-FU at a dose of 12 mg/kg ( a 50% reduction in the non-toxic dose) were evaluated to determine the the minimum 5-FU dose that will affect a very high MTX dose. Animals were observed for weight loss and mortality.

Cells and Measurement of Intracellular MTX and MTXPGs

MCF-7 breast cancer cells were grown in monolayer culture in Dulbecco's modified Eagles medium DMEM) containing 5% donor calf serum, 100 unit/ml of penicillin, 100 mg streptomycin, and 10 microgram/ml of insulin. Stock cultures were maintained in 75-$cm^2$ flasks and incubation at 37°C. Cell populations were serially passed every 3 to 5 days. Breast cancer cells were harvested with 5 ml of a trypsin-EDTA solution containing 0.5 g of trypsin and 0.2 g of EDTA/liter and 1 x $10^4$ cells were passed in 1 ml of DMEM at 1000 x g. Cells were grown for 72h in an atmosphere of 5% $CO_2$ in air (8), 0.7 $\mu$M MTX was added to the growing cells for 48h. MTX and MTXPGs were separated by using a paired ion HPLC system. Samples were injected onto a $\mu$-Bondapak $C_{18}$ reverse-phase column. Solvent A consisted of 10 mM potassium dihydrogen phosphate, pH 7.0 containing 10 mM tetrabutylammoniumhydrogen sulfate and solvent B consisted of methanol. The retention times of MTX and MTXPGs were determined the absorbance of the effluent the effluent at 254 nm. MTXPG2, MTXPG3 and MTXPG4 standards were custom synthesized by Moreavak Biochemicals, Brea, CA.

Results and Discussion

When cell extracts containing MTX were subjected to analysis by HPLC, peaks were detected which corresponded to standard MTXPG2, MTXPG3 and MTXPG4(8). At a cell density of $10^4$, the MTX, MTXPG2, MTXPG3, and MTXPG4 cellular levels, respectively, were 20.5, 7.3, 11.4, and 15.8. Since MTXPGs are in a greater amount than MTX, MTXPGs inhibitory effect should be sustained and longer than MTX as a result of MTXPGs cellular retention. Similar studies should be done to those which defined the relative amounts of MTX and MTXPGs (if any) in sensitive normal tissue such as bone marrow and gastrointestinal tissue. Figure 1 illustrates the effect of an acute administration of high dose MTX (250 mg / kg) and 5-FU (25 mg / kg) on bone marrow. A comparison is made between a priming dose of 5-FU administered 2 h before MTX vs. MTX only, 5-FU, and control bone marrow (myeloid : erythroid ratio). A marked increase in the myeloid : erythroid ratio occured in animals receiving MTX and 5-FU (2h) before MTX when compared only to 5-FU and control. However, a significant decrease in the myeloid : erythroid ratio occured when 5-FU (2h) before MTX is compared to MTX. There is no effect on peripheral blood from the acute administration of these agents.

A further manifestatioin of MTX toxicity is weight loss. To determine the lowest priming- and non-toxic dose of 5-FU which would have an effect on an extremely high dose of MTX, animals were administered 13 mg/kg of 5-FU and 1250 mg/kg of MTX. As shown in Fig. 2, extreme weight loss occured in all animals receiving MTX, with the most extreme loss occuring in MTX and MTX (2h) before 5-FU treated animals. In contrast to animals receiving only MTX or MTX (2h) before 5-FU, less weight loss results from the usage of a priming- and low non-toxic dose of 5-FU when given 2h before MTX administration. The maximum weight reduction after MTX, MTX (2h) before 5-FU, and a priming- and low non-toxic 5-FU dose 2h before MTX administration, respectively, is 3.5 g, 2.9 g, and 2.1 g. Whereas, animals treated with 5-FU alone loss only 0.3 g of body weight; and non-treated (saline-treated; control) animals loss no weight. All animals receiving 1250 mg/kg of MTX survived for 5 days.

**Conclusions**

The studies reported here indicate that the toxicity of very high dose MTX on bone marrow and animal body weight was greatest when MTX was administered alone and when MTX administration a non-toxic dose of 5-FU. These results suggest that the incidence and severity of MTX 2h before 5-FU toxicity is best related to MTX rather than 5-FU since 5-FU had no effect which differed significantly from control. A priming- and non-toxic-dose of 5-FU reverses high dose MTX acute effects on bone marrow. The erythroid cells 1) appear to be more sensitve than the myeloid cells to the acute adverse effect of MTX and 2) show marked protection by a priming and non-toxic dose of 5-FU. (This protection by 5-FU potentially could lead to an increase in quality of life in MTX-5-FU breast cancer protocols.)

Weight loss after treatment with MTX alone and MTX 2h before 5-FU may be associated with toxicity to the gastrointestinal tract. A 50% reduction in the priming-dose of 5-FU was effective in protecting, in part, against weight loss from an acute dose of MTX approximately 6 times the lethal dose. The limits of the lowest dose of 5-FU that has significant protection against the adverse effect of the highest dose of MTX has been established.

Cytotoxicity to MCF-7 human breast cancer cells may be the resultant of higher intracellular levels of MTXPGs than MTX. A determination of MTXPGs (if any) in MTX-susceptible normal cells in the presence and absence of a priming-dose of 5-FU would be of value the selectivity of MTX.

**References**


1. T.J. Robins, D. Bowen, Q.Q. Bui and M. Tran: Modulation of high-dose methotrexate toxicity by a non-toxic level of 5-fluorouracil. Toxicology 41:61-73, 1986.

2. T.J. Robins and D. Bowen: Toxicity, pathologic effects, and antineoplastic activity of a non-toxic dose of 5-fluorouracil in combination with methotrexate. Anticancer Research 8:43-50, 1988.

3. R. M. White, D. Bowen, R. Gumbs, and E. Myers: A phase I study of fluorouracil first followed by methotrexate: Dose intensity without toxicity. Proc. Am. Soc. Clin. Oncol. 10:111, 1991.

4. R. M. White: 5-Fluorouracil modulates the toxicity of high dose methotrexate. J. Clin. Pharmacol. 35:1156-1165.

5. G.P. Browman et.al.: Prospective randomized trial of one hour sequential versus simultaneous methotrexte plus 5-fluorouracil in advanced and recurrent squamous cell head and neck cancer. J. Clin. Oncol. 1: 787, 1983.

6. D. Bowen, E. Foelsch and L.A. Guernsey: The interaction between fluoropyrimidines and methotrexate, $^{14}$C-formate incorporation into nucleic acids and protein. Cancer Chemother. and Pharmacol. 4:111-116, 1980.

7. D. Bowen, B. D. Bailey and L.A. Guernsey: Rate-limiting steps in the interaction of fluoropyrimidines and methotrexate. European J. Cancer and Clin. Oncol. 20:651-657, 1984.

8. D. G. Kennedy et al.: The effect of the rate of cell proliferation on the synthesis of methotrexate polygamma glutamates in two human breast cancer cell lines. Biochem. Pharmac. 34:3087-3090, 1985.

ADDENDUM - J1

# A PHASE I STUDY OF FLUOROURACIL (FU) FIRST FOLLOWED BY METHOTREXATE(MTX):DOSE INTENSITY WITHOUT TOXICITY.

R. White, D. Bowen, R. Gumbs, and E. Myers. Howard University Cancer Center, Washington, D.C.

Laboratory models suggest that a priming dose of FU can modulate the toxicity of high dose MTX and maintain efficacy. The purpose of this study was to determine the maximum tolerated dose of MTX that can be given after a dose of FU without leucovorin (L.V.) rescue. 37 pts. (28M, 9F; 33 head and neck (HN), 1 each: lung, ovary, rectum, esophagus; Stage: 4 III, 33 IV; prior therapy: 15; 36 ambulatory) were entered. Cohorts of pts. received a constant dose of FU (500 mg/m$^2$) followed in 2 hrs by MTX, q 3 wk; urine was alkalinized prior to and for 24 hrs post MTX. Pts received MTX with 0 doses of L.V. If 5 and 2 doses of L.V. (10 mg/m$^2$ q 6 h) resulted in less than WHO Grade 2 toxicity. The results in previously untreated pts:

| MTX | L.V. | No. of | Median Nadir X1000/mm$^3$ | | Oral Toxicity No. of Courses | |
|---|---|---|---|---|---|---|
| mg/m$^2$ | Doses | Pts/Courses | WBC | Plat Ct | Grade: 1 | 3 |
| 200 | 0 | 2/2 | 6.75 | 295 | 1 | 0 |
| 400 | 0 | 6/7 | 4.1 | 228 | 2 | 0 |
| 700 | 0 | 7/17 | 4.9 | 228 | 2 | 1 |
| 1000 | 0 | 5/10 | 4.05 | 257 | 2 | 0 |
| 1250 | 0 | 6/12 | 5.8 | 251 | 4 | 0 |

Proc. Am. Soc. Clin. Oncol. 10:111, 1991

## Hematologic Toxicity in Previously Treated Subjects

| MTX Dose ($mg/m^2$) | LV Dose | No. of Subjects/ No. of Courses | Nadir Level (range) ($\times 10^9$ cells/L) | | % Hematocrit (range) |
|---|---|---|---|---|---|
| | | | WBC | Platelet Count | |
| 200 | 2 | 1/1 | 4.5 | 237 | 28 |
| | 0 | 3/6 | 4.0 (2.3–7.6) | 479 (73–543) | 30.5 (23.4–31.6) |
| 400 | 5 | 2/2 | 4.3 (3.1–5.4) | 307 (152–462) | 32.6 (30.4–34.8) |
| | 2 | 2/3 | 4.0 (3.3–4.7) | 175 (128–395) | 32.4 (23.3–34.7) |
| | 0 | 2/3 | 3.2 (2.6–3.4) | 411 (119–516) | 33 (26.6–34) |
| 700 | 5 | 2/2 | 4.5 (3.3–5.7) | 140 (138–142) | 31.1 (27.5–34.7) |
| | 2 | 1/1 | 6.2 | 142 | 34.3 |
| | 0 | 1/1 | 0.6 | 16 | 23.7 |

*MTX: methotrexate; LV, leucovorin, 10 mg/m² every 6 hours; WBC, white blood count; No. of newly enrolled subjects started at 0 LV step, 200 mg/m² MTX (3).*

## Nonhematologic Toxicity in Previously Untreated Subjects

| MTX Dose ($mg/m^2$)* | LV Doses | No. of Subjects/No. of Courses | No. of Oral Toxic Courses | | | No. of N/V Toxic Courses | | | No. of Skin Toxic Courses | | | Other (No. of Courses) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Grade 1 | Grade 2 | Grade 3 | Grade 1 | Grade 2 | Grade 3 | Grade 1 | Grade 2 | Grade 3 | |
| 200 | 5 | 3/3 | | | | | | | | | | |
| | 2 | 3/3 | 1 | | | | | | | | | alopecia (1) |
| | 0 | 2/2 | 1 | | | | | | | | | |
| 400 | 5 | 3/3 | | | | | | | | | | |
| | 2 | 3/2† | | | | | | | | | | |
| | 0 | 6/7 | 2 | | | | | | | | | |
| 700 | 5 | 3/3 | | | | | | | | | | |
| | 2 | 4/4 | | 1 | | | | | | | | scrotal cellulitis (1) |
| | 0 | 7/17 | 2 | 1 | | 1 | | | 1 | 1 | | alopecia (2) |
| 1000 | 5 | 3/3 | | | | | | | 1 | | | |
| | 2 | 3/3 | | | | | | | 1 | | | |
| | 0 | 5/10 | 2 | | | 2 | | | 3 | | | conjunctivitis (2) |
| | | | | | | | | | | | | alopecia (2) |
| 1250 | 5 | 5/5 | | | | | | | | | | sudden death (1) |
| | 2 | 5/5 | | | | 2 | 1 | | | | | |
| | 0 | 7/14 | 3 | 1 | 1 | 1 | | | 2 | | | renal (1) |
| | | | | | | | | | | | | cardiac (1) |
| 1600 | 5 | 6/6 | 1 | | | 1 | 1 | | 1 | | | |
| | 2 | 5/6‡ | 2 | 1 | 1 | 1 | | | | | | |
| | 0 | 3/3 | 1 | | | | | | | | | |

**No. of newly-enrolled subjects starting at the 0 doses of LV step: 400 mg/m² MTX (3); 700 mg/m² MTX (4); 1000 mg/m² MTX (2); 1250 mg/m² MTX (2).*

*N/V: nausea/vomiting.*

*† One subject refused follow up and the course is nonevaluable for both hematologic and nonhematologic toxicity. Subsequently, the subject had no toxicity in the 0 doses of leucovorin step.*

*‡ One subject started at this level and was dosed twice at this level.*

*MTX, methotrexate; LV, leucovorin, 10 mg/m² every 6 hrs.*

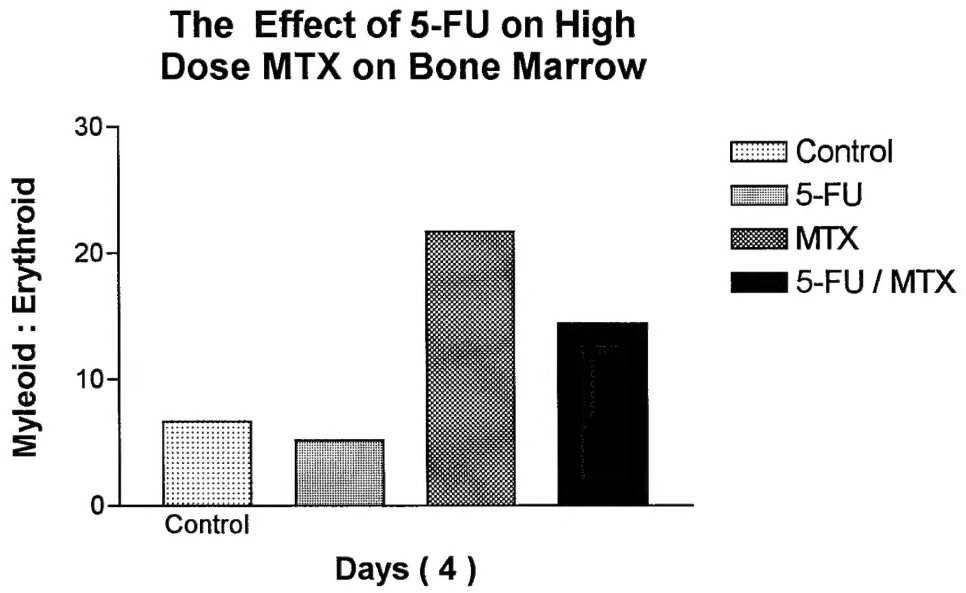


Figure 1. The effect of a (priming- and non-toxic-)5-fluouracil and high methotrexate dose on bone marrow.

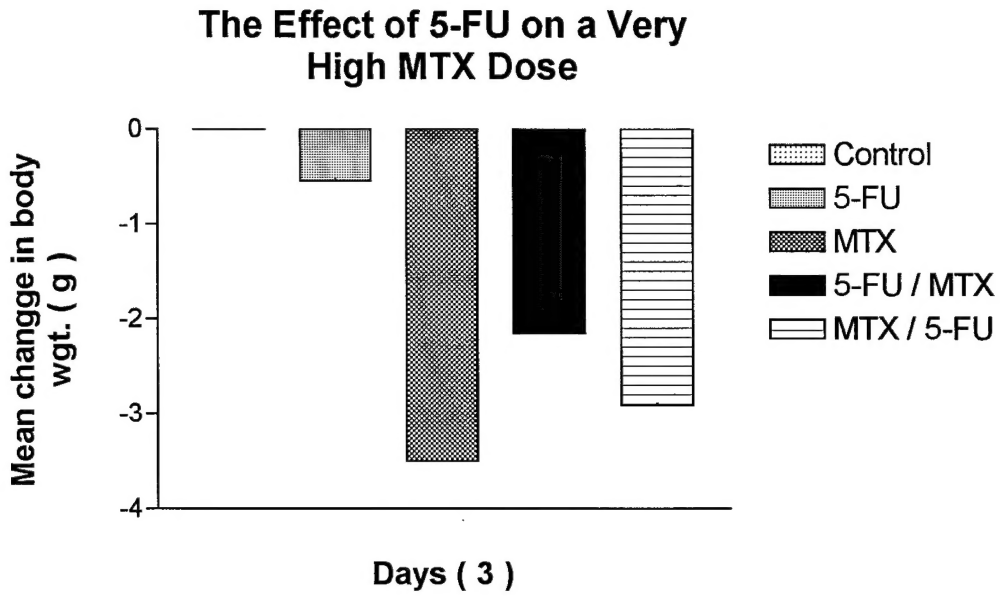


Figure 2. Changes in body weight associated with a low (priming-and non-toxic-) 5-fluorouracil dose and a very high methotrexate dose.